PROTOCOL

Protocol for GLP AOAC Use-Dilution Method
P2016

Test Microorganism(s)
Cronobacter sakazakii ATCC 29004

Product Identity
Test Substance: Sterilex Ultra Disinfectant Cleaner Solution 1
Lots: RS1-188A and RS1-188B

Test Substance Sterilex Ultra Activator Solution
Lots: RS1-190A and RS1-189B

Data Requirement
US EPA 40 CFR Part 158
U.S. EPA OCSPP 810.2200

Study Sponsor
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Protocol Number
P2016

Study Director
Kari Grant, B.S.

Date
14NOV2017

PROTOCOL (cont.)

I. Introduction

This document details the materials and procedure for evaluating the efficacy of liquid disinfectants using the AOAC Use-Dilution Method in accordance with Good Laboratory Practice Standards (GLPS) stipulated by 40 CFR 160. This document also explains the terms and conditions of testing.

II. Purpose

The purpose of this study is to document the efficacy of the test substance against the test system (microorganism) under the specified test parameters.

III. Justification for the Selection of Test System (Microorganism)

The test microorganism listed on page 1 of this protocol is designated as an additional claim microorganism for use in the AOAC Use-Dilution Method as well as per EPA Product Performance Test Guidelines, OCSPP 810.2200, Disinfectants for Use on Hard Surfaces, Efficacy Data Recommendations and other related EPA guidance.

IV. Terms and Conditions

Studies by Microchem Laboratory are conducted in accordance with general terms and conditions posted on www.MicrochemLab.com/terms.htm

Prior to study initiation, Microchem Laboratory must receive the approved and signed protocol, test substance and payment. Changes to the signed, approved protocol will require amendment and may incur additional fees. Cancellation of the study any time after the protocol has been signed will result in a cancellation fee of up to 100% of the total study cost, to be determined by laboratory management at its sole discretion.

Microchem Laboratory may repeat studies, free of charge, in the event of unintended protocol non-conformance, if the non-conformance is determined by the Study Director to have affected the study outcome. If the neutralization system specified for a study is not adequate, the study will be deemed "inconclusive" and the Study Sponsor will be responsible for the cost of the study. In addition, the Study Sponsor is responsible for the cost of all studies performed to confirm the outcome of a previous study and for ensuring that the study will meet their regulatory objectives.

The Study Sponsor must obtain written consent from Microchem Laboratory to use or publish its protocols, study reports (or parts thereof), logo or employee names for marketing purposes.

Test substance characterization as to content, stability, etc., is the responsibility of the Study Sponsor. The test substance shall be characterized by the sponsor prior to the completion of this study.

PROTOCOL (cont.)

V. Test Substance Identification, Characterization, and Handling

All test substances used to substantiate antimicrobial efficacy claims will be manufactured or otherwise tested at the lower certified limit (LCL).

Test Substance Name — **Sterilex Ultra Disinfecting Cleaner Solution 1**
Lot Number(s) — **RS1-188A**
Active Ingredients & Concentration — **Hydrogen Peroxide (6.14%), Quaternary Amine (5.81%)**
Manufacture Date — **07JUN2017**
Expiration Date — **07JUN2018**

Test Substance Name — **Sterilex Ultra Disinfecting Cleaner Solution 1**
Lot Number(s) — **RS1-188B**
Active Ingredients & Concentration — **Hydrogen Peroxide (6.03%), Quaternary Amine (5.82%)**
Manufacture Date — **07JUN2017**
Expiration Date — **07JUN2018**

Test Substance Name — **Sterilex Ultra Activator Solution**
Lot Number(s) — **RS1-190A**
Active Ingredient & Concentration — **N/A**
Manufacture Date — **07JUN2017**
Expiration Date — **07JUN2019**
Special Handling Requirements — None

Test Substance Name — **Sterilex Ultra Activator Solution**
Lot Number(s) — **RS1-189B**
Active Ingredient & Concentration — **N/A**
Manufacture Date — **07JUN2017**
Expiration Date —**07JUN2019**

Test substance characterization as to content, stability, etc., (40 CFR, Part 160, and Sub part F [160.105]) is the responsibility of the Study Sponsor. The test substance shall be characterized by the Sponsor prior to the completion of this study.

Test substances and devices are handled as follows:

- The test substance is stored at ambient (room) temperature under fluorescent lighting or in a cabinet.
- The test substance is shaken or otherwise mixed well immediately prior to use.
- The test substance is handled safely in accordance with the chemical risks it may pose, stated in the MSDS or by the Study Sponsor during the course of pre-study communication.

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VI. Study Parameters, Incorporated by Reference

Number of Tests Comprising the Study — **2 (1Test per Test Substance Lot)**
Carrier Type — **Stainless Steel Penicylinder**
Number of Carriers per Test Substance — **10 Carriers per Lot**
Test Substance Form — **Dilution Required (1:1:10). 1 part Sterilex Ultra Disinfectant Cleaner Solution 1 + 1 part Sterilex Ultra Activator Solution + 10 parts Diluent**
Test Substance Diluent — **400 ppm ± 10 ppm AOAC Hard Water**
Test Temperature — **20°C ± 1°C**
Organic Soil Load — **5.0% ± 0.1% (v/v) Heat Inactivated Fetal Bovine Serum**
Contact Time — **9 minutes ± 5 seconds**
Neutralization Broth — **Letheen Broth with 0.1% Catalase**

Proposed Experimental Start Date: **13NOV2017**
Proposed Experimental Termination Date: **15NOV2017**

VII. Test System (Microorganism)

Cronobacter sakazakii ATCC 29004

VIII. Materials

- Pure culture of the test system (microorganism).
- Pure culture of the reference microorganism.
- Sufficient quantity sterile 8 ± 1 mm od, 6 ± 1 mm id, 10 ± 1 mm length, type 304 stainless steel penicylinders free of visible flaws. For a 10 carrier test, at least 19 carriers are necessary per microorganism and lot of product tested (10 test carriers, 6 inoculum control carriers, 1 neutralization control carrier, and 1-2 viability control carriers). Extra carriers may be prepared for use in the study.
- Sufficient volume of reagent grade 1N NaOH solution.
- Sufficient quantity of clean, sterile 100 mm × 15 mm sterile Petri dishes.
- Sufficient quantity of sterile 9 cm Whatman #2 (or equivalent) filter paper rounds.
- Sufficient quantity of test tubes containing 10 ml sterile Tryptic Soy Broth.
- Sufficient volume of sterile deionized water.
- Sufficient clean, sterile 25 mm × 100 mm test tubes.
- Sufficient sterile tubes containing sterile phosphate-buffered saline, for dilution of microbial suspensions prior to plating.
- Sufficient volume of sterile Tryptic Soy Agar or other appropriate growth agar for enumeration of diluted microbial suspensions.
- Sufficient volume of Fetal Bovine Serum for addition of artificial "soil" load to microbial culture.
- Sufficient volumes of AOAC Hard Water preparation reagents (AOAC Hard Water Solution 1, AOAC Hard Water Solution 2, EDTA, Indicator Solution, Inhibitor Solution, Buffer Solution, and Sterile RO Water)
- Sufficient number of 25 mm × 150 mm test tubes containing 10 ml sterile subculture neutralization broth.
- Two or more bent wire transfer hooks of a type that can be flame-sterilized quickly yet are strong enough to fully support the weight of a penicylinder during transfer from one tube to the next.

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- Bunsen burner, microbiological incinerator, or micro-torch as appropriate to ensure rapid and complete flame-sterilization of transfer hooks.
- Sufficient quantity of micropipettes and appropriately sized sterile micropipette tips.
- Automatic pipettor (Pipet-Aid or similar) and various sizes of sterile serological pipets.
- Thermometer (for submersion in an equilibrated test tube to indicate the temperature of the test substance during the test).
- Incubator capable of sustaining temperatures of 36°C ± 1°C and 30°C ± 2°C.
- Forceps.
- Appropriate volume of 95% ethanol.
- Wire inoculating loop (4mm id).
- Sufficient number of test tube racks.
- Sonicator.
- Certified satellite clock.
- Certified digital timer.
- Water Bath capable of maintaining the appropriate test temperature.
- Sufficient quantity of appropriate antibiotic disks.
- Sufficient quantity of sterile swabs.

IX. Procedure

Preparation of AOAC synthetic hard water solution

- From each 1000 ml of sterile RO water (as measured by 1L volumetric flask), a volume equal to the total volume of AOAC hard water reagents added in the steps below is removed by serological pipette. For example, if 4 ml of solution "1" and 4 ml of solution "2" are to be added, then 8 ml of sterile water is removed.
- The concentration in PPM of hard water to be made is divided by 100. That is the volume, in ml, of AOAC hard water solution "1" will be needed to make 1000ml of hard water.
- Based on the calculation above, an appropriate volume of AOAC solution "1" is added to the sterile water, and mixed.
- The appropriate volume of solution "2" is then added and mixed.
- An appropriate volume of the synthetic hard water is removed and titrated. If necessary, the solution may be diluted with sterile water or augmented with parts of solution "1" and "2" to achieve the study sponsor requested hard water level. In any case, the hard water concentration of the final solution is to be determined by titration and recorded.

Preparation of Test Tubes and Test Substance

- Test substance is prepared by dilution in 400ppm ± 10ppm AOAC Synthetic Hard Water.
  - (1:1:10) by the addition of 1 part of Sterilex Ultra Disinfectant Cleaner Solution 1 to 1 part of Sterilex Ultra Activator Solution to 10 parts of AOAC Synthetic Hard Water.
    - For Lot: RS1-188A: 20.0ml of disinfectant solution, 20.0ml of activator solution, 203.57ml of hard water diluent.
    - For Lot: RS1-188B: 20.0ml of disinfectant solution, 20.0ml of activator solution, 202.63ml of hard water diluent.
- Test substance is used within 3 hours of preparation.
- Test tubes that will receive test substance are thoroughly cleaned and steam sterilized prior to use.

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- 10 ml of the prepared test substance is transferred by sterile disposable serological pipette, or other means as appropriate, into each 25 × 100 mm test tube designated for that purpose.
- Tubes containing test substance are equilibrated to test temperature for ≥10 minutes prior to initiating testing or recording test substance temperature.

Preparation of Test Tubes for Subculture/Neutralization and Incubation of Treated Carriers

- Before the test begins, the subculture/neutralization test tubes are prepared by cleaning, followed by the addition of 10 ml of an appropriate subculture neutralizing medium and steam sterilized prior to use.

Preparation of Test Carriers

- Before the test, clean stainless steel carriers are soaked in fresh 1M NaOH for at least 12 hours.
- Carriers are thoroughly rinsed using multiple tap-water rinses followed by a double R/O water rinse.
- An aliquot of rinse water from the final R/O water rinse is collected, and mixed with 2-3 drops phenolphthalein. If alkalinity is observed (rinse water turns pink) the carriers are re-rinsed until alkalinity is no longer observed.
- Carriers are distributed into an appropriate autoclavable container, covered with deionized or reverse osmosis water and steam sterilized.
- Carriers are allowed to cool to room temperature prior to use in the study.
- Prior to use in the study, carriers are observed for flaws and flawed carriers are discarded.

Preparation of Test Culture

- A daily culture of the test microorganism is created from the freezer or working stock culture in 10 ml Tryptic Soy Broth. This culture is incubated for 24 hours ± 2 hours at 30°C ± 2°C.
- Subsequent daily transfers (≤5) are made by transferring 0.010 ml of the most recent daily transfer culture into 10 ml Tryptic Soy Broth and incubated for 24 hours ± 2 hours at 30°C ± 2°C. Only one daily transfer is required prior to initiation of the final test culture.
- A test culture is initiated by transferring 0.010 ml of the most recent daily transfer culture into an appropriate number of test tubes, each containing 10 ml Tryptic Soy Broth and incubated for 48-54 hours at 30°C ± 2°C.
- Test cultures are vortex mixed and allowed to rest at ambient temperature for ≥ 10 minutes.
- The upper portion of the mixed culture(s) is removed, leaving behind any debris or clumps, and pooled in an appropriate vessel(s), if necessary.
- For the purpose of achieving carrier counts within the range of the study, dilution or concentration of the final test culture may be performed using the culture medium used to generate the test culture. Manipulated test culture is used within 30 minutes for carrier inoculation.

Supplementation of Test Culture with Organic "Soil" Load

- Thawed, sterile heat inactivated fetal bovine serum is added to the pooled test culture such that the final concentration is 5.0% ± 0.1% (v/v).
- The test culture-soil mixture is swirled gently to mix.

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Contamination of Carriers with Test Culture

- Deionized/reverse osmosis water is aspirated from the container containing the prepared carriers using a sterile serological pipette.
- The test culture is added to the drained vessel containing the penicylinders, such that all carriers are completely submerged in the test culture for uniform coverage (Approximately 1 ml of culture per carrier).
- The test culture and penicylinders are allowed to dwell for 15 minutes ± 2 minutes at room temperature.
- After 15 minutes ± 2 minutes have elapsed, the culture is aspirated and penicylinders are removed from the container aseptically using a sterile wire hook (carriers may be tapped or shaken prior to removal to remove excess culture) and are placed, no more than 12 carriers to a dish, on sterile double filter paper-lined, sterile Petri dishes. Carriers are placed on end, evenly spaced in the dish, such that they do not touch one another. If any carriers fall over, they are discarded from use in the test.
- Loaded Petri dishes are covered, transferred to an incubator at 36°C ± 1°C, and allowed to dry for 40 minutes ± 2 minutes or until visibly dry.
- Inoculated carriers are used within 2 hours of drying.

Exposure of Carriers to Test Substance

- Inoculated carriers are transferred, using a flame-sterilized wire hook, one carrier to each test tube containing 10 ml test substance, at appropriate intervals to ensure careful and aseptic handling. Every attempt is made to ensure that carriers are not allowed to touch the sides of the test tube during this step. If a contaminated penicylinder touches the sides of the test tube going into the test substance, then the corresponding test tube is noted.
- Tubes containing test substance and carrier are gently swirled then placed back in the water bath for the duration of the contact time.
- After the contact time for each carrier has elapsed, each carrier is removed from the test substance using a flame-sterilized wire hook. Carriers may be tapped in the lower third of the tube to remove excess test substance. Carriers are then transferred to a test tube containing 10 ml of the appropriate subculture/neutralization medium, such that it is completely submerged. If a treated penicylinder touches the sides of the test tube going into the neutralization/subculture media, then the corresponding tube is noted.
- Test tube racks are shaken and then incubated for 48 hours ± 2 hours at 30°C ± 2°C.
- After incubation, the number of test tubes showing growth is recorded.

Enumeration of Test Carriers

- Following the conclusion of the dry time, carriers are assayed in two sets of three; one set immediately prior to conducting the test, and one set immediately following the test. Each carrier is transferred individually to a subculture/neutralization test tube.
- These test tubes are placed in a beaker, filled with water to the level of liquid in the tubes, and held by hand in a sonicator so that the beaker bottom does not touch the bottom of the sonicator and all 3 liquid levels are approximately equal, and sonicated for 1 minute ± 5 seconds, timed with a certified digital timer.
- After sonication, the test tubes are pooled for each set of three carriers, serially diluted in sterile PBS and plated in duplicate within 2 hours of sonication using standard dilution and plating techniques.
- Enumeration plates are incubated for 48 hours ± 2 hours at 30°C ± 2°C.

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- The number of microorganisms present on the carriers after drying is determined using the following formula, including counts of "0," and excluding dilutions with counts of ">300." CFU = Colony Forming Units.

$$\frac{\text{(Average CFU for } 10^{-2}\text{)} + \text{(Average CFU for } 10^{-3}\text{)} + \text{(Average CFU for } 10^{-4}\text{)}}{10^{-2} + 10^{-3} + 10^{-4}} = \text{CFU/ml}$$

[(CFU/ml) × 10ml] = CFU/Carrier

NOTE: Other dilutions may be plated in the event that a different viable concentration is expected.

Neutralization Control

- A sterile uninoculated carrier is transferred to a test tube containing 10 ml of the test substance. After the specified contact time has elapsed, the carrier is transferred to a subculture/neutralization broth test tube, without allowing excess fluid to drain off of the carrier.
- After transfer, the test tube is inoculated with 10-100 CFU of test microorganism (obtained by serial dilution in PBS) and incubated along with the other test tubes. A parallel control tube containing only neutralizer is inoculated to verify growth of the target microorganism as a comparative control.
- The inoculum is plated in duplicate to verify the number of CFU added and incubated alongside the test.

Viability Control

- One to two inoculated test carriers are placed in individual subculture/neutralization broth tubes and incubated alongside the test.

Subculture/Neutralization Sterility Control

- A test tube containing only subculture/neutralization broth is incubated alongside treated carriers.

Carrier Sterility Control

- A sterile uninoculated carrier is added to a test tube containing subculture/neutralization broth and is incubated alongside treated carriers.

Test Microorganism Purity Control

- A volume of the test culture used in this study is subcultured onto growth agar medium and incubated alongside enumeration plates to morphologically confirm presence of a pure culture.

Media Sterility Controls

- A test tube containing only subculture/neutralization broth is incubated alongside the test to verify media sterility.
- A plate containing growth medium is incubated alongside the test to verify growth media sterility.
- A plate containing a volume of PBS is incubated alongside the test to verify enumeration diluent sterility.
- A plate containing a volume of Tryptic Soy Broth is incubated alongside the test to verify culture diluent sterility.

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- A plate containing a volume of AOAC Synthetic Hard Water is incubated alongside the test to verify test substance diluent sterility.
- A plate containing a volume of fetal bovine serum is incubated alongside the test to verify soil load sterility.
- Sterility controls are incubated for 48 hours ± 2 hours at 30°C ± 2°C.

Incubation of Tubes and Enumeration and Control Plates

- All tubes and plates are incubated at 30°C ± 2°C for 48 hours ± 2 hours.

Confirmation of Positive Tubes Following Incubation

- If multiple tubes demonstrate growth, ≥20% of those tubes are confirmed not to be a result of contamination by plating on growth media, or other analysis as appropriate.
- All confirmatory plates are incubated for 18-24 hours at 30°C ± 2°C.

X. Success Criteria

The experimental success (controls) criteria follow:

- The test microorganism must demonstrate a mean log density of at least 4.0 corresponding to 1×10^4 CFU/Carrier.
- The subculture/neutralization sterility control test tube is negative for growth.
- The carrier sterility control test tube is negative for growth.
- The viability growth control test tube(s) are positive for growth.
- The neutralization verification subculture/neutralization control and test tubes are positive for growth.
- The neutralization control inoculum demonstrates 10-100 CFU.
- The media sterility controls are negative for growth.
- The test microorganism purity control demonstrates no contaminant microorganisms.

The EPA performance criterion for disinfection follows:

- If 1 or more non-control subculture/neutralization test tubes are confirmed positive for *Cronobacter sakazakii* ATCC 29004 growth after incubation, then efficacy is not demonstrated by the test substance under the conditions evaluated.

Retesting guidance for disinfection follows:

- When a test fails and the $\log_{10}$ density of the test carriers is below 4.0, no retesting is necessary.
- When a test passes and the $\log_{10}$ density of the test carriers is below 4.0, retesting is necessary.

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XI. Reporting

- Results are reported accurately and fully, in accordance with EPA GLP (40 CFR Part 160). A draft report will be provided for review by the Study Sponsor prior to study completion.

XII. Data and Sample Retention

- The study report and corresponding data sheets will be held in the archives of Microchem Laboratory for at least 2 years after the date of the final report and then may be destroyed. If the study is used by the Study Sponsor in support of a label claim, documentation may be returned to the Study Sponsor for archiving at Study Sponsor's expense.
- The test substance may be returned to the Study Sponsor at Study Sponsor's request and expense within 30 days of study completion. If the Study Sponsor does not request return of the sample, it will be destroyed >30 days after study completion.

XIII. Quality Control

- The study is conducted in accordance with Microchem Laboratory's Quality Management System and will undergo a full quality assurance review. All protocol amendments will be fully recorded and reported, as well as any deviations from the protocol.

XIV. References

- 'Association of Official Analytical Chemists, International.' *AOAC Official Method 955.15.* Testing Disinfectants Against *Staphylococcus aureus*. Revised 2013.
- US EPA Product Performance Test Guidelines OCSPP 810.2200: Disinfectants for Use on hard Surfaces-- Efficacy Data Recommendations

XV. Protocol Approval

"I, the Study Sponsor, have read and understand the study protocol. By signing this protocol I am certifying that the information and parameters accurately describe the test(s) to be completed in accordance with Good Laboratory Practice Standards (GLPS) stipulated by 40 CFR 160. I have also read, understand and agree to the terms and conditions listed in the protocol."

Study Sponsor/Representative Signature Approving Protocol

John Reilly, Study Sponsor, Sterilex Corporation — Date: 11/15/17

Kari Grant, Study Director, Microchem Laboratory, LLC — Date: 17NOV2017

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